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# Glucose-6-phosphate phosphohydrolase activity in guinea pig liver microsomes is influenced by phosphatidylcholine. Interaction with cholesterol-enriched membranes

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**Guinea pig liver microsomal membranes were cholesterol-enriched by feeding guinea pigs a high-cholesterol diet. Cholesterol enrichment as well as partial lipid removal of normal native microsomes by acetone-butanol extraction resulted in 40–50% loss in activity of the glucose-6-phosphate phosphohydrolase (G-6-Pase) (EC 3.1.3.9) enzyme system. The activity was restored by supplementation of microsomal total phospholipid (PL) and its phosphatidylcholine (PC) species but not with microsomal neutral lipids, cholesterol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin or diphosphatidylglycerol (cardiolipin). The activity was decreased by sodium deoxycholate but enhanced by dimethylsulfoxide. Egg-yolk PC and asolectin influenced the activity of the enzyme to the same extent as microsomal PC did. Lipid depletion and cholesterol produced an increase in $K_m$ while the $V_{max}$ was lowered. The non-linearity in the Arrhenius plot of the native microsomes was lost on lipid removal and cholesterol enrichment. The energy of activation ($E_a$) calculated from the continuous line was found to be lowered to the level that was observed above the break points in intact microsomes. Addition of microsomal PC to the assay system decreased the $K_m$ of the enzymatic reaction in native membranes, in partially lipid-depleted and cholesterol-enriched membranes, but did not alter the $V_{max}$ values and only marginally influenced the non-linear relationship of the Arrhenius expression of temperature dependence. The ability of immature rat liver phospholipid exchange protein to introduce alien PL into microsomal membrane was used to study the lipid dependence of G-6-Pase. Protein-catalyzed and detergent (cholate)-mediated membrane PL exchange for egg-yolk PC from the PC/cholesterol unilamellar liposomes resulted in substantial loss of enzyme activity. The discrepancies in the influence of PC on G-6-Pase were interpreted by assuming that the enzyme was a two-component system, a surface-located substrate transporter unit and a membrane integral catalytic phosphohydrolase unit. The lipid microenvironment and PL requirement in particular, could be different for the two components, although they represented a single functional unit at the time of enzymatic reaction.**

## Introduction

Glucose-6-phosphate phosphohydrolase (G-6-Pase) (EC 3.1.3.9) catalyzes the terminal hydrolytic reaction of both gluconeogenic and glycogenolytic pathways and is an integral enzyme of the membranes of endoplasmic reticulum [1]. Arion et al. have established that the enzyme is a two-component system consisting of a glucose 6-phosphate (G-6-*P*)-specific transporter which mediated the movement of the hexose phosphate from the cytosol to the lumen of the endoplasmic reticulum and a non-specific phosphohydrolase-phosphotransfer-

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Abbreviations: ULV, unilamellar lipid vesicles; Chol, cholesterol; G-6-Pase, glucose-6-phosphate phosphohydrolase; PL-P, phospholipid phosphorus; PLEP, phospholipid exchange protein.

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ase localized on the luminal surface of the membrane [2]. Numerous membrane-bound enzymes have been found to have a functional dependence on the lipid bilayer related to changes in the physicochemical properties of the membrane lipid [3]. In an earlier publication, we have shown that G-6-Pase activity in rat brain microsomal vesicles can selectively be altered by partial lipid removal and resupplementation with endogenous or exogenous phospholipids (PL) in sonicated dispersions [4]. Detergent destabilisation and introduction of alien PL with the help of a non-specific lipid exchange protein, resulting in a changed lipid micro-environment in the membrane, have also altered the G-6-Pase activity. In these experiments, the zwitterionic PL, phosphatidylcholine (PC) is found to be most effective but it exerts completely different effects on the restoration of enzyme activity after partial lipid removal or even in native membrane. It stimulates the enzyme activity when added to the assay system as a sonicated dispersion but fails to do so or even decreases the activity in the modified membranes which received PC molecules under mild conditions in situ by earlier incubation with unilamellar lipid vesicles (ULV) and non-specific lipid exchange proteins. It is concluded that may be the two components of the enzyme system depend on different lipid micro-environments; the transporter unit being favourably stimulated by PC, while the more deeply membrane-buried hydrolytic part does not operate where the membrane lipid bilayer is unduly enriched with PC.

In the present paper, we have extended the investigation to liver microsomes of animals fed on a diet with high cholesterol (Chol) content, expecting that the introduction of cholesterol, even though in small amounts, into the membrane-lipid bilayer may impart restriction in mobility (or lateral diffusion) of one or both components of the enzyme. The Chol/PL molar ratio is the critical determinant of the membrane lipid ordering effect and as such this ratio will influence the membrane-bound enzyme activity. Using the same criteria as earlier, we have also examined the interaction of PC molecules with the increased lipid ordering in the membrane (increased Chol/PL molar ratio) and the resultant activity of the G-6-Pase enzyme system.

## Materials and Methods

*High cholesterol-diet feeding to the animals*

Mature male guinea pigs of 260–270 g body weight were obtained from the Institute's inbred colony and housed in the laboratory-attached animal house for 1 month for thorough acclimatization. The animals were fed stock pellet diet (Lipton India Ltd.) and water ad libitum. Eight animals in each group were then fed for five months either a diet containing 225 g butter, 13 g cholesterol and 9 g cholic acid per 4.5 kg diet amounting to 20% added fat intake or the stock diet containing 5% fat. The average food intake was found to be 30 g per animal per day. The body weights and serum cholesterol values of the animals were recorded before commencement and after the termination of the experiment. Overnight fasted animals were killed under ether anaesthesia around 10 a.m. to avoid diurnal variation and the livers were further processed after brief perfusion with ice-cold normal saline.

*Preparation of microsomal vesicles*

The livers were excised and placed in ice-cold buffer containing 250 mM sucrose, 5 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid): 1 mM dithiothreitol and 0.5 mM EGTA, pH 7.0 (buffer A). The minced livers were washed with cold buffer, suspended in 2 vols. of buffer and homogenized by four passes at 500 rpm in a Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was diluted with buffer to give a 10% homogenate. Mitochondria, nuclei and intact cells were sedimented by centrifugation for 10 min at $8000 \times g$. Any unsedimented mitochondria and lysosomes were removed by centrifugation of the supernatant at $15000 \times g$ for 10 min. Microsomes were sedimented by centrifugation of the supernatant from the later spin, at $113000 \times g$ for 60 min. The pellets were suspended in 0.25 vol. of 150 mM Tris-HCl (pH 8.0) and microsomes were resedimented by centrifugation for 30 min at $113000 \times g$ [5]. The pellets were suspended in buffer A, at a final protein concentration of 30–40 mg/ml.

The homogeneity of microsomal membrane preparations was tested by assaying marker enzymes. High specific activity of G-6-Pase and NADPH–cytochrome-*c* reductase [6] was detected in the preparation while the extent of mitochondrial contamination did not exceed 5–6% as determined from succinate dehydrogenase activity [7]. Integrity of the microsomal vesicle preparations was adjudged by the latency of mannose-6-phosphate phosphohydrolase activity [8]. Only those preparations with latencies of 95% or more were used.

*Microsomal disintegration and reconstitution*

Partial delipidation of microsomes was carried out by a modification of the method of Fiscus and Schneider [9] as adopted by Chu and Rooney [10], where chilled acetone (2 ml) was added to about 6 mg of microsomal protein in an ice-water bath. After centrifugation at $115000 \times g$ for 10 min at 4°C the pellet was re-extracted with chilled 1-butanol (2 ml) and centrifuged again under the same conditions. The solvent was evaporated completely under a stream of $N_2$ and the pellet was finally suspended in buffer A.

Lipids wherever mentioned, were added to the enzyme assay system from chloroform/methanol (2:1, v/v) solution in desired concentrations after completely

removing the solvent under a stream of $N_2$, adding appropriate amount of enzyme assay buffer and then strongly sonicated in an ice water and $N_2$ environment. Auto-oxidation of PL was prevented by addition of 0.01% butylated hydroxytoluene.

*Assay of G-6-Pase activity*

G-6-Pase activity was measured by a modification of the method of Zakim and Vessey [11]. Microsomes (0.1 mg protein) were incubated in a total volume of 0.5 ml in 100 mM sodium acetate and 20 mM glucose 6-phosphate (G-6-*P*) (monosodium salt), pH 6.5, containing 10 mg/ml fatty acid free bovine serum albumin with or without 0.4% sodium taurocholate. Samples were kept on ice for 30 min prior to the addition of G-6-*P* to initiate the reaction. Samples were then transferred to a shaking water bath at 37°C. The reaction was stopped after 30 min by chilling on ice and adding 0.5 ml of 10% chilled trichloroacetic acid. The tubes were centrifuged and the phosphohydrolase activity was measured in the supernatant from the $P_i$ released. Standards and blanks were analysed under identical conditions except that no microsomes were included. In experiments measuring latency of the phosphohydrolase to mannose 6-phosphate (M-6-*P*), G-6-*P* was replaced by 2 mM M-6-*P*.

*Partial purification of PL-exchange protein*

Extracts of whole liver possessing PL-exchange activity from immature rats were prepared following the first several stages of the Crain and Zilversmit [12] procedure for isolating non-specific PL-exchange proteins from bovine liver, as described by us earlier [4,13]. This included: (1) sequential centrifugation at $13000 \times g$ for 30 min and at $105000 \times g$ for 60 min of a 35% homogenate of the tissue in 0.25 M sucrose 0.07 M Tris-HCl (pH 7.0), 0.001 M EDTA strictly at 4°C, with discarding of the resulting pellets, (2) precipitation of the cytosol at pH 5.1 by adjusting with 3 M HCl, the mixtures being allowed to stand for 2 h at 4°C and then centrifuging at $15000 \times g$ for 15 min, the precipitate being discarded and the pH of the supernatant obtained, readjusted to 7.4 with solid Tris, (3) ammonium sulfate precipitation between 40 and 90% of saturation, with 15 min of stirring each time between 0–40 and 40–90% of salt saturation and centrifugation at $10000 \times g$ for 30 min, and (4) dialysis against 3 mM sodium phosphate, 5 mM $\beta$-mercaptoethanol and 0.02% $NaN_3$ (pH 7.4). PL-exchange activity was well preserved on storage in this medium at −20°C. The protein was found to possess the capability of transferring $^{32}$P-labelled PL including PC between mitochondria and microsomes, $^{3}$H-labelled glycosphingolipids between liposomes and erythrocytes and $^{14}$C-labelled cholesterol between liposomes and intestinal brush-border membrane vesicles as established earlier by using non-exchangeable lipid marker [13,14].

*Analytical procedures*

Microsomal protein was measured by the modified sodium dodecylsulfate-Lowry procedure of Lees and Paxman [15] using bovine serum albumin as the standard. Microsomal lipids were extracted by the method of Folch et al. [16]. Neutral, phospho- and glycolipids were separated in batch by silicic acid column chromatography following the procedure of Vance and Sweeley [17]. PL were separated by thin-layer chromatography on silica gel 60 plates with chloroform/methanol/aqueous ammonia (65:25:4, v/v) and the lipid-bound phosphorus was quantitated by the method of Bartlett [18]. Cholesterol was measured following the procedure of Zak [19]. Inorganic phosphorus produced by phosphohydrolase activity on G-6-*P* was measured by the method of Chen et al. [20].

*Preparation of unilamellar lipid vesicles (ULV)*

Small ULV were prepared freshly by dissolving egg-yolk PC and cholesterol (Sigma Chemical Co.) (1:0.7, mol/mol) with butylated hydroxytoluene (0.01% (w/w) of PC), as an antioxidant in chloroform, in a round bottom flask. These lipids gave single spot when checked by thin-layer chromatography, at least in two different solvent systems. The lipid mixture was flushed with $N_2$ and evaporated to dryness in a rotary evaporator. This was redissolved in diethyl ether and subsequent evaporation resulted in a thin lipid film on the wall of the flask. Buffer B (155 M NaCl, 8.1 mM $Na_2HPO_4$, 1.9 mM $NaH_2PO_4$, 0.3 mM $Na_2$EDTA (pH 7.4) and 2 mg glucose per ml) was added to give final dispersion of less than 10 mg lipid per ml. The flask was swirled by hand until all lipids were freed from the sides of the flask and large lipid aggregates were dispersed. The milky suspension so formed was briefly agitated using a vortex mixer and then allowed to stand for 2 h at room temperature. This was sonicated with a probe type sonicator with a microtip (MSE) for 30 min in an ice-water and $N_2$ environment. Following sonication, this was centrifuged for 1 h at $140000 \times g$. Poorly dispersed lipids, titanium metal emanating from the sonicator probe and large multilamellar particles were pelleted while the slightly translucent supernatant was used as ULV in the experiments [21].

*Protein-catalyzed exchange of PL between ULV and microsomes*

Microsomes (2–6 mg protein, 1000–3000 nmol of PL) were incubated for 60 min at 37°C in a total volume of 1 ml in 250 mM sucrose, 0.5 mM Hepes, 1 mM dithiothreitol and 0.5 mM EGTA (pH 7.0), containing 10 mg of fatty acid free bovine serum albumin (Cohn fraction V, Sigma Chemical Co.). Microsomes were incubated in the presence or absence of PL-exchange protein (3–7 mg protein) and in the presence of ULV (2-fold molar excess of PL to limit the back

exchange). After 60 min of incubation, lipid transfer was stopped by adding 2 ml of ice-cold buffer and placing the samples on ice. ULV were separated from the microsomes by centrifugation for 30 min at 103000 $\times g$. The microsomal pellet was homogenized by hand with a Dounce type all glass homogenizer in 3 ml of buffer A and G-6-Pase activity was estimated [22].

*Detergent-mediated exchange of microsomal PL for synthetic PC*

The detergent-mediated PL exchange procedure of Warren et al. [23] was performed with the following modifications. 3.9 mg of asolectin was suspended by sonication in a solution containing 10 mM Tris-$H_2SO_4$ (pH 7.5), 1 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 100 mM sucrose and 5.8 mM sodium cholate. After addition of the microsomes (2 mg protein), the mixture (0.3 ml final volume) was incubated for 90 min at 30°C under $N_2$. Separation of unbound PL and detergent from the microsomes was accomplished by centrifugation at 105000 $\times g$ for 90 min at 30°C. The pellet was suspended in buffer A and G-6-Pase activity was estimated.

*Phospholipase C treatment of microsomes*

Microsomes (1.4 mg of protein, 2800 nmol of PL) were incubated with phosphatidylcholine-specific phospholipase C (10 mg) from *Clostridium perfringens* (Sigma Chemical Co.) at 25°C for 1 h in 1 ml of 0.15 M NaCl adjusted to pH 7.4 with $NaHCO_3$, but without calcium. $Ca^{2+}$, which is known to activate phospholipase C, was avoided in our system as addition of $Ca^{2+}$ might also cause aggregation of microsomes as well as inactivation of G-6-Pase and other enzyme systems. The reaction was terminated by addition of EDTA, at a final concentration of 5 mM, thereafter cooled on ice and subsequently analysed [24].

*Measurement of apparent $K_m$, $V_{max}$ and energy of activation ($E_a$)*

The measurement of apparent $K_m$ and $V_{max}$ values of G-6-Pase in the normal and high cholesterol-fed intact and partially deplipidated microsomes was carried out at eight different substrate concentrations in the 10– 100 nM range and by constructing a double-reciprocal Lineweaver-Burk plot from the data [25]. The enzyme activity was also determined in the same microsomal preparations at a fixed substrate concentration over a range of temperature between 18 and 45°C and ln $V$ was plotted against $1/T$ ($T$ is the absolute temperature in K) to obtain the Arrhenius expression of temperature dependence of G-6-Pase. $E_a$ was calculated from the slope of the line by the equation: slope = $(-E_a/2.303) \cdot R$, where $R$ represents the gas constant [26].

## Results

*Quantitative differences in lipids of normal and high cholesterol-fed liver microsomes*

Liver microsomes from guinea pigs fed normal and high-cholesterol diets were found to differ by their PL compositions and PL/Chol molar ratios (Table I). The ratio was found to be lowered from 7.5 in rats fed normal diet to 4 in microsomal membranes of high-cholesterol diet fed animals. The difference was, however, not due to contamination with other subcellular compartments, as established by marker enzyme assays. The ratio of PL/Chol was a major determinant of membrane microviscosity and therefore might influence the membrane functions such as membrane-associated enzyme activities [3]. There was also an increased quantity of PL in the cholesterol-fed membranes. Compositional difference in the PL in the two membranes was, however, not evident. PC constituted the bulk of total PL, 50.2%, while the next most abundant PL was phosphatidylethanolamine, 21.7% of the total PL. Other PL species which occupied the membrane in descending order were, phosphatidylinositol 4.7%, lysophosphatidylethanolamine 3.9% and diphosphatidylglycerol (cardiolipin) 2.2%.

TABLE I

*Phospholipid composition of the microsomal membranes from normal and high-cholesterol fed guinea pig liver* [a]

| | Normal | High-Chol-fed |
|---|---|---|
| Lipid (nmol/mg protein) | | |
| Cholesterol | | |
| Total | 97.96 | 323.01 |
| Free | 48.77 | 163.29 |
| Esterified | 49.01 | 160.03 |
| Phospholipid | | |
| Total | 679.55 | 1358.36 |
| PL/Chol (molar ratio) | 7.02 | 4.21 |
| Phosphatidylserine | 57.11 | 115.32 |
| Phosphatidylinositol | 31.32 | 63.28 |
| Sphingomyelin | 38.49 | 77.25 |
| Phosphatidylcholine | 340.51 | 679.23 |
| Phosphatidylethanolamine | 147.52 | 294.45 |
| Lysophosphatidylcholine | 21.31 | 43.38 |
| Lysophosphatidylethanolamine | 26.29 | 52.38 |
| Diphosphatidylglycerol (cardiolipin) | 14.61 | 29.35 |
| Body weight on high-Chol diet feeding (g) | | |
| Initial | 260–275 | – |
| Terminal | 296 | 370 |
| Serum cholesterol on high-Chol diet feeding (mg/dl) | | |
| Initial | 109.33 | – |
| Terminal | 100.80 | 245.33 |

[a] The data presented here and in the following tables represent an average of two independent experiments, determined in duplicate and the individual data varied by less than 5% from the average.

TABLE II

*Effects of microsomal lipids added in sonicated dispersions on glucose-6-phosphate phosphohydrolase activity in native and partially delipidated microsomes of normal and high-cholesterol fed guinea pig liver*

| Addition (1 μM) | Glucose-6-phosphate phosphohydrolase relative activity (% of control) | | | |
|---|---|---|---|---|
| | normal | | high-Chol fed | |
| | native | acetone-butanol extracted | native | acetone-butanol extracted |
| None | 100 [a] | 66 | 79 | 57 |
| Total neutral lipid | 96 | 67 | 72 | 51 |
| Cholesterol | 90 | 67 | 70 | 50 |
| Total phospholipid | 364 | 183 | 281 | 149 |
| Sphingomyelin | 116 | 62 | 89 | 45 |
| Phosphatidylserine | 115 | 62 | 88 | 55 |
| Phosphatidylinositol | 106 | 68 | 92 | 65 |
| Phosphatidylcholine | 392 | 242 | 279 | 157 |
| Phosphatidylethanol-amine | 105 | 60 | 87 | 43 |
| Diphosphatidylglycerol | 102 | 50 | 95 | 48 |
| Asolectin | 393 | 190 | 240 | 173 |
| Egg-yolk phosphatidyl-choline | 390 | 180 | 260 | 160 |
| Dimethylsulfoxide (1% v/v) | 208 | 179 | 187 | 115 |
| Sodium deoxycholate | 89 | 51 | 60 | 40 |

[a] 100% is 83.15 nmol/min per mg protein.

*Effect of microsomal lipids on G-6-Pase*

Microsomal G-6-Pase activity was assayed in fresh intact microsomes, in microsomes partially dilipidated by acetone-butanol extraction and in conditions where PL had been added to the enzyme assay system in sonicated dispersions. Feeding a high-cholesterol diet to the animals had resulted in 21% loss of enzyme activity (Table II). Partial lipid removal causes 33 and 22% loss of the activity compared to the intact membranes in normal and high-cholesterol fed animals, respectively. Microsomal lipids were isolated from the respective membranes by chromatographic procedures and added to the enzyme assay system in sonicated dispersions. Use of total lipid, total PL and neutral lipids, cholesterol, individual phospholipid fractions and two exogenous PL, besides sodium deoxycholate and dimethylsulfoxide (DMSO) in the reconstitution process allowed detailed evaluation of the nature of lipid requirement for complete expression of G-5-Pase activity. Both in the intact as well as acetone-butanol extracted membranes, addition of microsomal PL and its PC species markedly increased the enzyme activity. The exogenous PC species such as egg-yolk PC and asolectin also caused marked elevation in the enzyme activity in both kinds of membranes and in their acetone-butanol extracted fractions. The effects were nearly identical to that of the effects produced by microsomal PC. Deoxycholate caused a marginal reduction in enzyme activity while the elevation in G-6-Pase activity by DMSO was quite pronounced.

*PL-mediated modification of kinetic properties of G-6-Pase*

Influences of PC supplementation on the kinetic parameters of enzymatic reaction were studied from the Lineweaver-Burk double-reciprocal expression. Acetone-butanol extraction resulted in 54.64 and 75.03% increase in apparent $K_m$ compared to the intact membranes in normal and high-Chol fed microsomes, respectively (Table III). There was also an increase noted, although marginal, in the $K_m$ value of high-Chol fed intact membranes when compared to the normal ones. Partial lipid removal resulted in substantial reduction in $V_{max}$ values in both kinds of membranes. Intact membranes, enriched with cholesterol also showed reduced apparent $V_{max}$ of G-6-Pase when compared with the normal microsomes. PC supplementation resulted in the substantial recovery of the activity in all the cases.

*Temperature dependence of G-6-Pase*

Effect of temperature on G-6-Pase activity was studied from the Arrhenius expression and revealed that only the enzyme activity of intact microsomes showed a non-linear temperature response while acetone-butanol extracted membranes exhibited a continuous Arrhenius plot and an $E_a$ which was increased to the value usually

TABLE III

*Effects of partial delipidation and supplementation with phosphatidylcholine (PC) on the kinetic and thermodynamic parameters of glucose-6-phosphate phosphohydrolase activity of normal and high-cholesterol fed guinea pig liver microsomes*

| Kinetic/ thermodynamic parameter | Normal | | | | High-Chol fed | | | |
|---|---|---|---|---|---|---|---|---|
| | native | + PC | acetone-butanol extracted | + PC | native | + PC | acetone-butanol extracted | + PC |
| $K_m$ (nM) | 1.63 | 1.23 | 4.16 | 3.11 | 1.92 | 1.02 | 7.69 | 5.05 |
| $V_{max}$ (nmol·min$^{-1}$·(mg protein)$^{-1}$) | 111.1 | 128.5 | 66.6 | 101.23 | 58.8 | 98.75 | 12.5 | 73.56 |
| $E_a$ (kJ·mol$^{-1}$) | 34.9 | 27.1 | 51.4 | 31.5 | 51.6 | 35.6 | 65.2 | 39.3 |

obtained with intact microsomes above the break points. Addition of PC to the intact and disrupted microsomes lowered the energy of activation (Table III). In the cholesterol-enriched intact microsomes, the break in the Arrhenius plot could no longer be observed and a constant $E_a$ was measured over the whole temperature range, which again came close to the value found above the break point in native microsomes.

*Effect of membrane modification on G-6-Pase activity*

In order to substitute a part of the microsomal membrane PL for exogenous PC and to see the effects thereupon on G-6-Pase enzyme system, the membranes were incubated at 22°C for 1 h with the ULV prepared from egg-yolk PC and cholesterol (10 : 1, mol/mol) and a phospholipid exchange protein (PLEP), partially purified from immature rat liver post-microsomal pH 5.1 supernatant. Membrane modification was also performed through cholate-mediated exchange of membrane PL for synthetic PC, by incubating the microsomes with sonicated egg-yolk PC or asolectin and sodium cholate and then removing the detergent by centrifugation. As presented in Table IV, these techniques produced changes, albeit small, in membrane composition as monitored by phospholipid phosphorus (PL-P)/protein ratio before and after the incubation. There was only marginal change in PL-P/protein ratio in membranes before and after incubating with PLEP and sodium cholate. However, in both cases, G-6-Pase activity was lowered significantly, amounting to 30–40% of reduction as compared to the native and unmodified intact microsomes, from normal as well as high-cholesterol fed animals. Selective hydrolysis of membrane PL was also achieved by mild treatment of microsomes with PC-specific phospholipase C from *Clostridium perfringens* which is known to hydrolyse selectively the PL present on outer surface only [27,28]. Phospholipase C treatment resulted in more than 60% reduction in the activity of G-6-Pase (Table IV).

## Discussion

The challenge of the present study is to critically anatomize the lipid microrequirement of the microsome-bound G-6-Pase enzyme system. Often, conflicting results have been provided on the lipid dependence of this enzyme for either its regulation or expression [29]. This is primarily because of the complexity of approaches employed in studying the lipid-enzyme association, the variety of techniques used in membrane modifications and the contradictory interpretations arising from thereon [5]. We have reexamined the problem by following the enzyme activity after mild partial lipid depletion and reconstitution with sonicated dispersion of endogenous or exogenous PL. Membrane modification is also effected by introducing alien PL with the help of PLEP or detergent and assaying the enzyme activity in the modified membrane system thereupon. Interaction of the PL-enzyme complex with cholesterol has also been studied by producing cholesterol-enriched microsomes after prolonged feeding of the animals with high-cholesterol diet.

In our experiments, PC emerges to be the PL species that stimulates G-6-Pase system when added in sonicated dispersion in the disintegration-reconstitution procedure. PC also does so in the high cholesterol-en-

TABLE IV

*Effects of phospholipid exchange protein and detergent (cholate)-mediated phospholipid exchange on the glucose-6-phosphate phosphohydrolase activity of normal and high-cholesterol fed guinea pig liver microsomes*

Phospholipid phosphorus (PL-P) and protein values are expressed in nmol and mg, respectively, while G-6-Pase enzyme activity is presented in nmol substrate hydrolyzed per min per mg of protein.

| Enzyme system | Normal | | | | | | High-Chol fed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | native | | | acetone-butanol extracted | | | native | | | acetone-butanol extracted | | |
| | PL-P/protein | | G-6-Pase | PL-P/protein | | G-6-Pase | PL-P/protein | | G-6-Pase | PL-P/protein | | G-6-Pase |
| | before incubation | after incubation | | before incubation | after incubation | | before incubation | after incubation | | before incubation | after incubation | |
| Microsomes | 707 | – | 82.32 | 501 | – | 44.09 | 908 | – | 68.13 | 701 | – | 30.97 |
| + PC-Chol liposomes and phospholipid exchange protein | 705 | 700 | 38.71 | 503 | 508 | 19.2 | 908 | 807 | 35.54 | 706 | 700 | 14.72 |
| + PC and sodium cholate | 701 | 608 | 36.58 | 409 | 503 | 22.49 | 900 | 905 | 37.28 | 709 | 707 | 13.76 |
| + phospholipase C | 709 | 306 | 32.43 | – | – | – | – | – | – | – | – | – |

riched membranes, thus overcoming the stiff energy barrier provided by the cholesterol (possibly by causing steric hindrance to the neighbouring PL fatty acid molecules). These results are in complete agreement with the reports of Garland et al. [30,31] where the solubilized G-6-Pase has been shown tc be reactivated by PC. In hepatomas, where the microsomes frequently show a lower PC to phosphatidylethanolamine ratio [32], also shows very low G-6-Pase activity [33]. In the deoxycholate-disrupted hepatoma microsomes also, addition of PC causes activation of G-6-Pase while other PL have only little effect [34]. The reduction in enzyme activity by phospholipase C treatment of the microsomes, which is both PC-specific and is known to selectively hydrolyze the outer surface PL, provides support to the dependence of G-6-Pase on this particular PL species, seemingly for the substrate transport activity. However, when PC is introduced in the membranes, native or cholesterol-enriched, via PLEP or cholate mediation, the enzyme activity is greatly diminished. The present set of data confirms our earlier observations on the rat brain microsomai G-6-Pase enzyme system, that PC which constitutes nearly 50% of the microsomal PL is critically involved in stabilizing the enzyme and more so for that part of the enzyme which is involved in transporting the substrate and channelizing it to the lumenal space of the endoplasmic reticulum, to be acted upon by the hydrolytic component of the enzyme. It further confirms that the functional G-6-Pase enzyme is a two component system, consisting of a superficial transporter protein and a vectorial catalytic part of the enzyme which is deeply buried into the hydrophobic interior of the membrane. Accordingly, the lipid requirement of the two components may also be different. The addition of PC in sonicated dispersion in the assay system can enrich the microenvironment of the enzyme where the transporter is operating to bring G-6-*P* in, while the introduction of this PL in the lipid bilayer suppresses the catalytic phosphohydrolase unit of the enzyme in some unknown way. Use of protein-catalyzed exchange of PL in comparison with detergent mediated PL exchange between lipid vesicles and biological membranes such as microsomes is a simple uncomplicated method and allowed modifications of lipid bilayer in situ under a very mild condition without possibly affecting the organization of the enzyme protein and therefore fully allows the study of lipid dependence of G-6-Pase activity in almost native environment. Zilversmit and Hughes [35] and Van den Besselaar et al. [36] have shown that in rat liver microsomes, PC which comprises nearly 50% of the total PL, is rapidly exchangeable as a single kinetic pool.

Deoxycholate and DMSO have been used to evaluate the effects on G-6-Pase expression by non-lipid compounds. Deoxycholate at the concentration used, probably removes a substantial portion of membrane PL beyond the critical limit, causing a net reduction in enzyme activity. DMSO increases the enzyme activity in a completely different way as compared to the PL, by its capability to remove the permeability barrier of the enzyme towards its substrate and it also increases the aqueous solubility and critical micellar concentration of certain lipids [37]. DMSO is also a known water structure breaker [38] and, therefore, may disrupt the thickness of the unstirred water layer and in the process facilitates to bring the substrate closer to the membrane-bound enzyme [38].

The ability of monomeric cholesterol to produce functional changes in integral proteins of biological membranes is well established [39,40]. The introduction of this compound in liver microsomes in our study is achieved through high-cholesterol diet feeding, which permits a better understanding of its association with membrane PL to influence the G-P-Pase system. The rigidity imparted by this compound in the membranes is reflected in the restriction of the enzyme to be fully expressed. PC introduced into the system in sonicated dispersion is found to overcome the microviscosity barrier due to cholesterol, at least partly, a fact which may serve some pointer in the molecular understanding of the atherosclerotic processes. However, the real dilemma posed in our results is that the introduction of egg-yolk PC into the membrane lipid bilayer of cholesterol-enriched microsomes markedly reduces the enzyme activity. Possibly this is so because egg-yolk PC is highly enriched in saturated fatty acids such as palmitic acid and relatively low in long chain polyunsaturated fatty acids and therefore, may cause less fluidization. Study of the isolated enzyme protein in a reconstituted lipid vesicle (liposome) system with defined compositions in respect of fatty acid chain length, unsaturation, PL/Chol molar ratio, PL headgroups and associated charges, and PL endothermic phase transition characteristics can provide more definitive information regarding the effect of membrane lipid compositions on microsomal G-6-Pase enzyme, provided it is possible to present both transporter and catalytic component of the enzyme as a single topographical unit in the lipid vesicle.

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